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Genetická struktura mediteránních populací kaloně *Rousettus aegyptiacus*

Genetic structure of Mediterranean populations of fruit bat *Rousettus aegyptiacus*

Diplomová práce

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Prohlášení:

Prohlašuji, že jsem diplomovou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena pro získání jiného nebo stejného akademického titulu.

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Abstract

The genus *Rousettus* represents the only fruit bat genus distributed both in Asia and Africa reaching northern distributional limits of the Pteropodidae family. This unusual distribution pattern is related to the ability of echolocation, subsequent cave dwelling and probably other thermoregulatory and behavioural adaptations to relatively cold and dry climate. Methods for identification of genetically discrete populations were used in the presented study to acquire better comprehension of historical ways of colonization along with current dispersal and migratory patterns of the Egyptian fruit bat (*Rousettus aegyptiacus*) in the Mediterranean basin and adjacent range patches. Modern approaches to population and landscape genetics were applied on a dataset comprising 553 individuals from 72 localities using 20 nuclear microsatellites. Our results revealed a significant genetic distance of East African individuals and certain substructure in the northern part of the range. Cypriot population is clearly separated, and - for higher K - the isolation of colonies from Egyptian oases is highly supported. Genetic proximity of south Arabian and Sinai populations contradict current taxonomy of the species. Our findings highlight the role of seas and deserts as barriers restricting gene flow and the evolution of the population substructure within fruit bats, which is also verified by biogeographic patterns known in the family from other areas. The possibility of nascent island speciation in *Rousettus aegyptiacus* on Cyprus is also considered.

Key words: *Rousettus aegyptiacus*, Egyptian fruit bat, population structure, Mediterranean, landscape genetics, microsatellites

Abstrakt

Rod Rousettus představuje jediný rod kaloně, který se vyskytuje v Asii i v Africe a dosahuje severního okraje areálu čeledi. Toto neobvyklé rozšíření je spojeno se schopností echolokace, následným obýváním jeskyní a pravděpodobně s dalšími etologickými a termoregulačními adaptacemi k životu v poměrně chladném a suchém klimatu. Pro tuto studii byly použity metody k rozpoznání jednotlivých geneticky odlišných populací za účelem lepšího pochopení cest kolonizace a současného disperzního a migračního chování kaloně egyptského (*Rousettus aegyptiacus*) v Mediteránních a jim přilehlých oblastech. Moderními postupy populační a krajinné genetiky bylo analyzováno 553 jedinců ze 72 lokalit za použití 20 jaderných mikrosatelitů. Naše výsledky ukázaly významnou genetickou vzdálenost jedinců z východní Afriky a další dělení v severní části areálu. Výrazně se oddělují kyperské populace a pro vyšší K se dále vymezují kolonie z egyptských oáz. V rozporu s dosavadní taxonomií druhu je genetická blízkost populací z jihu Arabského poloostrova a ze Sinajského poloostrova. Naše zjištění upozorňují na roli mořských a pouštních oblastí jakožto bariér zabraňujících genovému toku a na vývoj populační struktury u kaloňů, která je v souladu s biogeografickými schématy známými v rámci dané čeledi i v dalších oblastech. Je rovněž zvažována možnost začínající ostrovní speciace kaloně egyptského na Kypru.

Klíčová slova: *Rousettus aegyptiacus*, kaloň egyptský, populační struktura, Mediterán, krajinná genetika, mikrosatelity

1 Introduction

1.1 Fruit bats

The term Flying foxes commonly used for fruit bats approximates well the appearance of these exceptional mammals. Most of autapomorphies of this group are related to their unusual diet, which is strictly phytophagous, containing nectar, fruit, pollen or leaves of a wide range of plant species (Fujita and Tuttle 1991). Besides, the localization of suitable food is facilitated via olfaction and vision while these senses are of lesser importance for bats of other families which orientate primarily by echolocation. The body size ranges from very little forms like *Balionycteris maculata* measuring around 6 cm from head to toe to very large ones such as *Acerodon jubatus* which is considered the largest known bat in the world with its wing span of up to 1.7 m (Nowak 1999). Although the main habitat for most of the nearly 200 species lays in the tropics and subtropics of the Old World, the fruit bat we focus on has penetrated remarkably far to the north, where climate conditions vary during the year. Sadly, the formerly abundant animals are almost everywhere threatened by people. As well as other rainforest dependent creatures, the fruit bats roosting in trees of these forests are jeopardised by increasing forest destruction. Furthermore, there is much more danger arising from humans to all bats including uncontrolled use of insecticides and bat hunting for food or to eradicate them from plantations (Amr et al. 2006; Fujita and Tuttle 1991). A recent event in Lebanon, where thousands of Egyptian fruit bats were killed by vandals is just an another example of the on-going threat (Alabaster 2012).

In accordance with the latest taxonomy (Hutcheon et al. 1998; Springer et al. 2001; Teeling et al. 2005), the Pteropodidae family together with Rhinolophidae, Hipposideridae, Megadermatidae, Craseonycteridae and Rhinopomatidae, is a member of the Yinpterochiroptera suborder of the Chiroptera order. The internal phylogenetic relationships of the order have been rearranged many times in the past and are of a big concern among zoologist all over the world. Contentious work built primarily on neuroanatomical traits and musculoskeletal adaptations denied the monophyletic origin of bats while other authors never doubted that all

Chiropterans share a common origin and disproved the purported paraphyletic hypothesis (Pettigrew et al. 1984 contra Ammerman and Hillis 1992; Kirsch et al. 1995; Thewissen and Babcock 1991). Based on the evaluation of flight apparatus and dental characteristics, two suborders - Microchiroptera and Megachiroptera - had subsisted and the Pteropodids had formed the only family in the latter one (Koopman 1994; Simmons 1998). However, the most recent subdivision of the order was not realized until after the expansion of molecular biology. Although relationships within the fruit bat family have also undergone several revisions (Giannini et al. 2006; Giannini and Simmons 2003; Juste et al. 1999; Romagnoli and Springer 2000), a final cladistic classification has not been finished yet. The most complete analysis on the grounds of huge amount of genetic data and a very precise methodical treatment provided a resolution of six fundamental clades and a detached lineage within the family. All these clades (Cynopterinae, Harpyionycterinae, Nyctimeninae Macroglossini, Epomophorinae + Rousettini, Pteropodini + *Melonycteris*) and the subfamily of single genus *Eidolon*, no matter whether they were new or previously proposed, were statistically proved as monophyletic. Unfortunately, the relationships among these lineages seem to be difficult to assess and may suggest a basal furcation of the Pteropodidae (Almeida et al. 2011).

1.2 The Egyptian fruit bat

Distributed throughout south Asia, Africa and Near East, ten different species of the *Rousettus* genus (Gray 1821) are described to date. Four of them are island endemic. *R*. *bidens* and *R*. *linduensis* live on Sulawesi, *R*. *madagascariensis* inhabits Madagascar and *R*. *obliviosus* is found on Comoros only (Simmons 2005).

Six geographically corresponding subspecies of *Rousettus aegyptiacus* (Geoffroy 1810) are recognized so far: *R*. *a*. *arabicus*, *R*. *a*. *aegyptiacus*, *R*. *a*. *leachii*, *R*. *a*. *princeps*, *R*. *a*. *tomensis* and *R*. *a*. *unicolor* (Bergmans 1994; Juste et al. 1996; Kwiecinski and Griffiths 1999)[1].

[1] In the original studies referred to as *Rousettus egyptiacus*.

As a fruit bat, Egyptian *Rousettus* feeds on fruits, flowers or leaves of various plants depending on their availability, for example rubber tree (*Ficus elastica*), Persian lilac (*Melia azadirachta*), carob (*Ceratonia siliqua*), apple (*Malus* sp.), fig (*Ficus carica*), pomegranate (*Punica granatum*) and many others (Albayrak et al. 2008; Benda et al. 2007). It dwells in caves thanks to its echolocation ability, which is exceptional among bats for the sound being emitted by tongue vibrations (Nowak 1999). Despite the well-developed eyesight characterizing all fruit bats, these animals stay active during the night and rest at their roost for most of the day (Korine et al. 2004). In relation to geographical latitude, representatives of this species breed any time during the year (closer to the equator) or in clear breeding seasons (further from the equator). The reproduction ethology embraces miscellaneous pre-copulation rituals of impressive acoustic, visual and olfactory effects. There is usually a single offspring in a birth (Horáček 1986; Nowak 1999). The wide distribution of the species, as illustrated in Fig. 1, is reaching the northernmost margin of the range of the whole family and points to some unusual skills these fruit bats must have. Their occurrence in the relatively harsh region is thought to be connected with the emergence of cultivation of agricultural crops by humans and thus provision of food supply (Galil et al. 1976, as cited in Korine et al. 1999). Even though hibernation is not developed, thermoregulatory and behavioural adaptations have improved for the survival in fairly dry savannahs and temperate climate zones (Noll 1979).

Fig. 1 Map of geographic range of Rousettus aegyptiacus

Original map source: The IUCN Red List of Threatened Species. Available at: http://www.iucnredlist.org

Rousettus aegyptiacus is a broadly studied model species. Research of this animal probably embraces all imaginable areas of study including its morphology, physiology, behaviour, ecology or agricultural impact since it is being considered a pest in several countries. Yet its largely used taxonomy published in 1994 by Bergmans was based on morphology and has not been confirmed by molecular records until the present time. In many cases observed polymorphisms are very beneficial but their connection to genealogical relationships is often masked by phenotypic plasticity. The development of molecular markers for the purpose of investigating genealogies and consequently phylogeography of not only mammals helps researchers to evaluate previous judgments by knowledge acquired from DNA. Calculations of allele frequencies for pooled samples (like populations)

and the opportunity to discriminate between homozygotes and heterozygotes is now possible by co-dominant markers which provide locus-specific information. Recent events causing evolutional changes are easily detected when highly polymorphic microsatellite loci are examined. The mutation rate of microsatellites is generally even higher than the rate of mitochondrial genes, which mutate faster than an overall nuclear DNA and are suitable for tracking moderately distant evolutionary events to the past. Allozymes, mtDNA sequences or nuclear microsatellites are common tools for obtaining information on population ecology from studied organisms (Freeland et al. 2011). The existing population studies of the Egyptian fruit bat in the Middle East were only partially successful in resolving clear structure by means of mitochondrial genes, which enforced the need of employing faster evolving markers for such research. Although mtDNA sequence divergence is shallow, a number of private haplotypes in some populations was found and landscape genetics analysis sustained the existence of spatially determined clusters (Benda et al. 2007; Benda et al. 2012 in prep.; Dundarova 2011). Besides, enormous differences in body dimensions occur over the whole area thought to be populated by a single subspecies of a fruit bat. Intraspecific phylogeny studied in insular populations of *Rousettus aegyptiacus* has already upheld recognition of respective populations as discrete subspecies through allozymes (Juste et al. 1996).

1.3 The aims

The aim of this thesis is to obtain and analyse genotypic data based on 20 nuclear microsatellite loci and to infer yet unknown population specifics of *Rousettus aegyptiacus* in the given region. Firstly, I focus on descriptive characteristics of each colony, relationships among them and their geographical distribution. Secondly, I investigate the presence of population substructure in the studied territory by dint of individual based approach along with landscape genetics methods and correlate the outcomes with geographic features of the region. Finally, my goal is to interpret generalized resulting hypotheses in connection with historical and ecological aspects and to outline further potential analyses.

To achieve a better comprehension of historical ways of colonization along with current dispersal and migratory patterns, we apply the recognition of genetically discrete populations. Regarding bats, it could be theoretically expected that population forming is low as a result of their flight capacity and that species are panmictic within the species range. However, this assumption is seldom verified empirically, as there are often important influences which hinder random mating such as complicated migratory and mating behaviour connected with site fidelity, physical obstructions to gene flow and other key aspects (Burland et al. 2001).

More detailed knowledge of the overall biology and ecology of the Egyptian fruit bat will enable us to introduce reliable scientific reasons for its conservation in order to protect these unique bats as they are the only frugivorous chiropterans in the Palearctic region (Horáček 2000).

Exhaustive information on fine scale biogeography and biology of *Rousettus aegyptiacus* in the Mediterranean cannot be gathered merely from the genetics. Hence, this paper creates a part of a much bigger project and concerns only certain aspects. Data obtained in consequence of thorough field investigations performed by other participants are not to be discussed here.

In this thesis I initially describe the methodology of the project and present the main outcomes. These are followed by discussion, where I intend to integrate our results with the existing background evidence regarding my issue and to offer some further implications of this study. Finally, I summarize all important facts and key hypotheses for the reader in the Conclusions section.

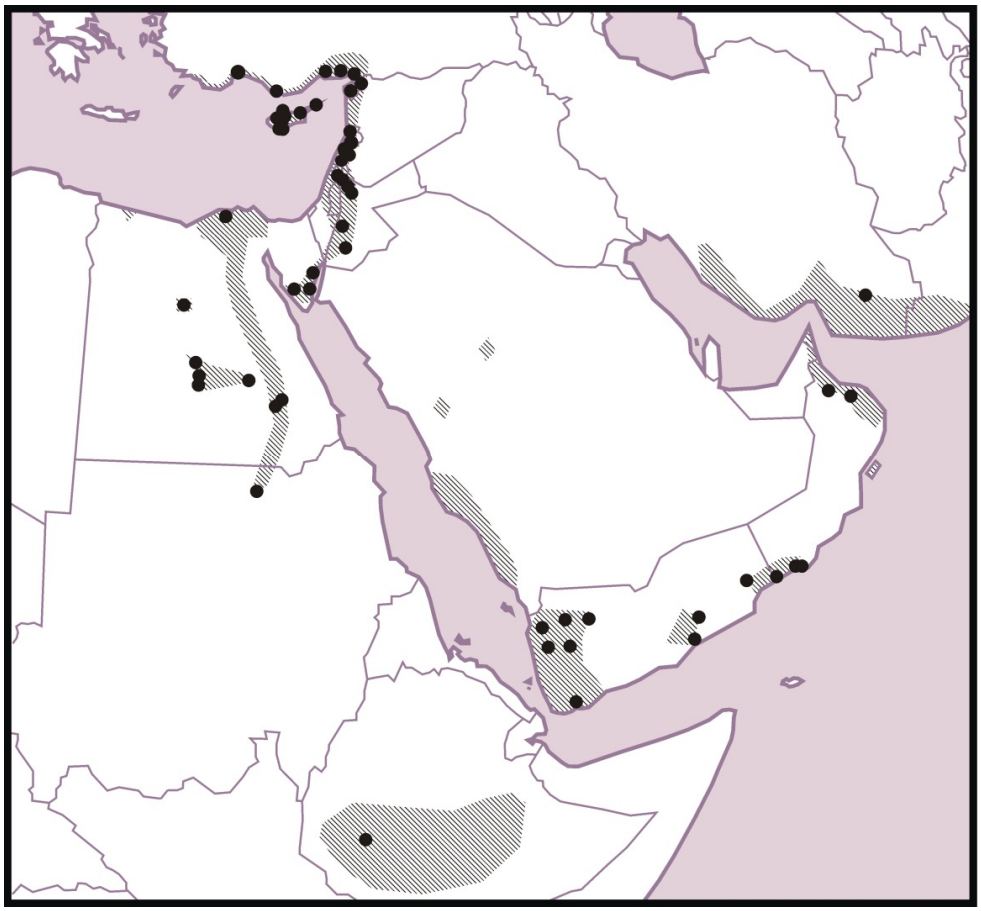
2 Materials and methods

2.1 Sampling and deoxyribonucleic acid (DNA) extraction

The study is based on 553 individuals sampled throughout Mediterranean region, Arabian peninsula, Iran and East Africa. The collection of samples was held during expeditions from 2005 to 2011. All 72 localities were referenced in geographic coordinate system (see Fig. 2). By application of mist netting, populations were mostly sampled near cave entrances, abandoned buildings or rock crevices where they roost, close to their feeding sites or at flying corridors. Plagiopatagium biopsies (Wilmer and Barratt 1996) or cells from buccal swabs were preserved in 96 per cent ethanol and stored at -20°C in laboratory after transportation. Additional 22 specimens from Prague ZOO were included forming a complete dataset of 575 individuals.

Fig. 2 Map of sampling sites

Black dots show the sampling sites, hatched pattern refers to the species extant range. Few locations in East Africa (Malawi, Uganda) are not depicted due to space limitations



Purification of total genomic DNA from both buccal swabs and tissue samples was performed according to standard protocols with QIAGEN DNeasy Blood & Tissue Kit or Macherey Nagel Nucleospin. DNA concentration was then measured on spectrophotometer ND-1000 (Nanodrop) and diluted accordingly to standardized values.

2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was conducted for an amplification of 20 microsatellite loci (described in Tab. 1) using primers previously designed for *Rousettus madagascariensis* (Andrianaivoarivelo et al. 2008) and *Rousettus leschenaulti* (Hua et al. 2006). Multiplex PCR Kit (Qiagen) was used following the manufacturer's instructions. Four groups of labelled primers were set together and PCR was carried out separately according to annealing temperatures and size ranges. Details on multiplex PCR cycles are provided in Tab. 2; Tab. 3 defines the content of PCR reaction mix. Precise volume for each primer was set on the basis of test reactions and every multiplex primer mix was then refilled up to 250 µl by TE buffer (Tab. 4). Thermocycler iCycler Thermal Cycler (BIO–RAD) was used for all the reactions performed.

Tab. 1 Description of microsatellite loci

Locus name	GenBank	Repeat motif	k	HW	Size range	Source species	Label	T_{an} (°C)
66HDZ343	EU884014	$(CA)_{22}$	16	***	113–148	*Rousettus madagascariensis*[2]	VIC®	58
66HDZ407	EU884016	$(GT)_2$ $(GA)_3$ $(GT)_{15}$	18	***	129–173	*Rousettus madagascariensis*	NED™	56
66HDZ327	EU884008	$(GT)_3$ $(GT)_{19}$	13	***	132–166	*Rousettus madagascariensis*	PET®	50
66HDZ106	EU884002	$(CA)_{21}$	12	***	166–194	*Rousettus madagascariensis*	6FAM™	56
66HDZ110	EU884003	$(GT)_{24}$	18	***	178–220	*Rousettus madagascariensis*	VIC®	54
66HDZ413	EU884017	$(CA)_{21}$	15	***	205–300	*Rousettus madagascariensis*	NED™	54
66HDZ340	EU884012	$(CA)_{14}$	18	NS	125–161	*Rousettus madagascariensis*	PET®	60
66HDZ117	EU884004	$(CA)_{18}$	18	***	154–194	*Rousettus madagascariensis*	NED™	60
66HDZ80	EU883997	$(GT)_{14}$	17	***	174–212	*Rousettus madagascariensis*	VIC®	58
66HDZ334	EU884009	$(CA)_{17}$	12	***	171–199	*Rousettus madagascariensis*	6FAM™	60
66HDZ105	EU884001	$(CA)_{13}$	16	*	190–226	*Rousettus madagascariensis*	PET®	58

[2] Andrianaivoarivelo et al, 2008

66HDZ341	EU884013	$(CA)_9$ CG $(CA)_{13}$	14	***	233–268	*Rousettus madagascariensis*	NED™	58
66HDZ82	EU883998	$(CA)_{26}$	21	***	226–280	*Rousettus madagascariensis*	VIC®	60
66HDZ304	EU884006	$(GT)_{22}$	20	***	146–192	*Rousettus madagascariensis*	6FAM™	62
M3-121	DQ389100	$(GT)_{15}$	21	***	149–203	*Rousettus leschenaulti*[3]	PET®	65
M3-6	DQ389097	$(GT)_{16}$	24	***	155–202	*Rousettus leschenaulti*	VIC®	65
M3-8	DQ389096	$(GT)_{20}$	12	***	146-170	*Rousettus leschenaulti*	NED™	65
M3-120	DQ389099	$(CA)_{13}$	15	***	173–210	*Rousettus leschenaulti*	6FAM™	61
66HDZ20	EU883996	$(GT)_{20}$	17	***	183–224	*Rousettus madagascariensis*	PET®	62
M3-1	DQ389102	$(CA)_{14}$ C $(CA)_2$	16	***	191–220	*Rousettus leschenaulti*	VIC®	61

HW - goodness-of-fit Hardy-Weinberg equilibrium test

k – number of alleles detected

NS – not significant

*** - statistical significance P<0.001

*- statistical significance P<0.05

Tab. 2 Settings for PCR thermocycler

		Multiplex		
		1a, 1b	2a, 2b	3a, 3b
Cycle	**Step**	**Temperature (°C); Time (min)**		
1 (1×)	Pre-denaturation	95; 15	95; 15	95; 15
2 (30×)	Denaturation	94; 0.5	94; 0.5	94; 0.5
	Annealing	54; 1.5	59; 1.5	62; 1.5
	Extension	72; 1	72; 1	72; 1
3 (1×)	Final elongation	60; 30	60; 30	60; 45
4 (1×)	Preservation	20; ∞	20; ∞	20; ∞

[3] Hua et al, 2006

Tab. 3 Composition of the reaction mixture

	Multiplex			
	1a, 1b	2a, 2b	3a	3b
	Volume (µl)			
PCR master mix	5	5	5	5
Primer mix	2×0.8	2×1	1	1
Rnase-free H_2O	2.4	2	3	3
DNA (c=0,1-10ng/µl)	1	1	1	1
Total volume	10			

Tab. 4 Volumes of primers

Set	Locus name	Primer (F and R)[4] V (µl)	Set	Locus name	Primer (F and R) V (µl)
Multiplex 1a	**66HDZ343**	1.65	**Multiplex 2b**	**66HDZ105**	6.65
	66HDZ407	5.00		**66HDZ341**	5.00
	66HDZ327	1.65		**66HDZ82**	1.50
Multiplex 1b	**66HDZ106**	2.50	**Multiplex 3a**	**66HDZ304**	1.65
	66HDZ110	1.65		**M3-121**	2.50
	66HDZ413	2.50		**M3-6**	1.65
Multiplex 2a	**66HDZ340**	6.65	**Multiplex 3b**	**M3-8**	1.65
	66HDZ117	1.65		**M3-120**	1.65
	66HDZ80	0.75		**66HDZ20**	3.00
	66HDZ334	1.13		**M3-1**	2.50

[4] Volumes apply for both forward and reverse primers.

Fluorescently labelled PCR products in 2 µl volumes were mixed with 7.5 µl formamide and 0.5 µl size standard (Gene Scan™ 500 LIZ Size Standard, Applied Biosystems). After denaturation at 95°C for 5 minutes DNA fragments were separated by capillary electrophoresis on a 3130xl Genetic Analyser (Applied Biosystems) with polymer POP-7 (Part No. 4352759) and standard DS-330 in sequencing laboratory of Biological Science section at Charles University in Prague.

2.3 Microsatellite data analysis

Raw allele sizes were read in microsatellite genotyping software GeneMarker V1.91 (SoftGenetics, State College, PA, USA). Raw data were binned and formatted in AutoBin software (Guichoux et al. 2011, http://www4.bordeaux-aquitaine.inra.fr/biogeco/Ressources/Logiciels/Autobin). All allele sizes of microsatellite loci employed in the study may be found in part I of the Appendix.

All individuals were grouped according to their origin into 72 putative populations while specimens from the Prague ZOO constituted one single population. A list of the populations along with Global Positioning System (GPS) coordinates of their localities may be found in part III of the Appendix. Due to the necessity of certain minimal number of individuals comprised in a population needed for statistical calculations within population genetics approach, only the populations of at least 5 individuals were analysed in corresponding programs. The occurrence of genotyping errors including null alleles, stuttering and large allele drop out was tested using a Monte Carlo simulation of expected allele-size differences by Micro-Checker 2.2.3 (Oosterhout et al. 2004). These errors could easily deform outputs from subsequent analytical procedures. Basic descriptive characteristics of investigated loci were generated by Cervus 3.0 (Kalinowski et al. 2007) and FSTAT 2.9.3.2 (Goudet 1995). FSTAT was used for the estimation of inbreeding coefficient (F_{IS}), gene diversity (h; Nei 1973), allelic richness (AR) and allelic diversity. The data were scanned in Cervus for possible matching genotypes (identity analysis), then estimations of expected heterozygosity (H_E) and observed heterozygosity

(H_O) for determined populations were calculated. As inbreeding increases the proportion of homozygotes in population, F_{IS} measures the reduction in heterozygosity compared to heterozygosity that would be expected in a randomly mating population with the same allele frequencies. Negative F_{IS} values mean that H_O is higher than expected, F=0 stands for no inbreeding, whereas F=1 means that population consists only of homozygotes (possible complete inbreeding). Gene diversity, allelic richness, allelic diversity and observed/expected heterozygosity are all measures of genetic diversity, although some are sensitive to sample size. R_{ST}, Theta and PhiPT represent types of F-statistics, developed originally by Wright (1951), which quantifies the genetic differentiation between populations (Freeland et al. 2011). Pairwise PhiPT (Φ_{PT}) were calculated among populations by program GenAlEx 6.41 (Peakall and Smouse 2006).

The subpopulation pattern within the investigated area was inferred using an individual based Bayesian clustering method in Structure 2.3.2 (Falush et al. 2007), separate runs were averaged by Structure-sum version 2011 (Ehrich 2006) and results graphically displayed in Distruct (Rosenberg 2004). All individuals could be included in the dataset for Structure, as the implemented algorithm treats every genotype independently. Two datasets were made. The first encompassed all our samples; genotypes from Prague ZOO were excluded in the second. Parameters were set as follows: 10 replicate runs for each K were conducted for K=1 to K=10, burn-in period consisted of 10000 steps (first 10000 steps to be discarded), 1000000 steps were set for collecting data. Admixture model was applied, population of origin was not used as prior information and the option of uncorrelated allele frequencies was chosen. The best K was determined by calculating Delta K (Evanno et al. 2005), lnP(D) - the estimated log probability of data and clusteredness in the program Structure-sum.

To gain a better view on how genetic variability is distributed spatially, the program GeneLand (Guillot et al. 2005) was employed for analysing data with landscape genetics approach. The implemented model is looking for within group Hardy-Weinberg and linkage equilibrium. First 1000000 MCMC (Markov chain Monte Carlo) iterations were run five times to determine the most suitable number of clusters with the following settings: thinning of 100

(the proportion of MCMC iterations saved), K values from 1 to 10, uncorrelated allele frequency model, noise blurring of coordinates of 5 km. Afterwards, a model with the same parameters except for ten times more iterations, burn-in of 10000 and K derived from initial screening was run. Results were displayed as maps of posterior probabilities of population membership. Genetic discontinuities uncovered by the procedure were linked to potential geographic barriers by visual comparison with maps of the region. To fulfil the assumptions of the method, the dataset was reduced to consist only of samples from continuous area covering the Mediterranean populations for this analysis.

The relationship between geographic and genetic distances among populations was examined by Isolation By Distance Web Service (IBDWS Version 3.22) (Jensen et al. 2005). The Geographic Distance Matrix Generator 1.2.3 (Erst 2011) computed spatial distances among localities for this testing. For each population pair the genetic distance was estimated both as F_{ST} using the methods of Weir (1990) and Rousset's distance measure ($F_{ST}/(1-F_{ST})$). Results were plotted and a Mantel test (Manly 1994) assessed whether the correlation between the pairwise genetic distance matrix and the pairwise geographic distance matrix is significant. The slopes and intercepts were calculated using reduced major axis (RMA) regression and confidence intervals were generated based on several different assumptions regarding data structure. Only colonies of at least five fruit bats appeared in the test and also those from Prague ZOO were omitted because of their unknown origin.

3 Results

3.1 Basic characteristics of microsatellite loci and populations

Studied microsatellite loci exhibit between twelve and twenty four alleles (average seventeen). Nineteen loci out of twenty are significantly in Hardy-Weinberg equilibrium (HWE); no loci show evidence for null alleles (Tab. 1).

The elemental characteristics of populations with five or more individuals involved are summarized in Tab. 5.

Amongst populations, observed heterozygosity (H_O) extents from 0.425 to 0.687 and expected heterozygosity (H_E) from 0.554 to 0.674; there is no considerable distinction between the two values for each population. The gene diversity (h), which is almost unaffected by sampling effects and in a population in HWE equivalent to H_E, ranges from 0.555 to 0.742; values very close to H_E calculations. The inbreeding coefficient is generally very low (average 0.051) indicating low level of mating between relatives. Allelic richness (AR) and allelic diversity (A) range from 1.554 to 1.742 and 3.4 to 6.95 respectively. The examined populations comprise 6 to 45 individuals from natural colonies in Cyprus, Turkey, Lebanon, Jordan, Egypt and Oman. Fruit bats from Prague ZOO are assembled in an artificial population with unknown origin.

Tab. 5 Descriptive characteristics of populations with ≥ 5 individuals

Country	Locality	h[5]	Ng[6]	A[7]	AR[8]	H_O[9]	H_E[10]	F_{IS}[11]
Cyprus	Ahanas, Androlika	0.574	16	4.3	1.574	0.574	0.574	-0.004
Cyprus	Pissouri	0.600	12	3.7	1.592	0.473	0.592	0.210
Cyprus	Ergates - at fruiting date palm	0.576	14	3.9	1.574	0.539	0.574	0.040
Cyprus	Gerolakkos - Alaykoy, gallery in quarry	0.588	8	3.4	1.579	0.461	0.579	0.204
Cyprus	Mammari	0.584	25	4.7	1.582	0.505	0.582	0.147
Cyprus	Afendrika	0.599	8	3.8	1.599	0.586	0.599	0.025

[5] Gene diversity
[6] Number of individuals in the population
[7] Allelic diversity
[8] Allelic richness
[9] Observed heterozygosity
[10] Expected heterozygosity
[11] Inbreeding coeficient

Cyprus	Yedikonuk	0.603	13	4.2	1.600	0.563	0.600	0.064
Cyprus	Smigies	0.555	8	3.5	1.554	0.530	0.554	0.048
Turkey	Antalya, gardens at suburb	0.674	16	5.1	1.674	0.672	0.674	-0.006
Turkey	Adana, old flour factory	0.642	17	5.2	1.642	0.640	0.642	-0.003
Turkey	Sayköy	0.672	19	5.6	1.671	0.656	0.671	0.012
Turkey	Cevlik, cave above village	0.665	9	4.8	1.666	0.674	0.666	-0.002
Turkey	Demrek, Dipsiz cave	0.646	16	5.2	1.647	0.687	0.647	-0.073
Turkey	Harbiye, cave in travertine in city	0.660	15	5.2	1.659	0.636	0.659	0.022
Lebanon	Adloun	0.635	8	4.3	1.636	0.645	0.636	-0.025
Lebanon	Bergual cave	0.621	10	3.5	1.622	0.425	0.622	0.283
Lebanon	Jeita	0.649	20	5.2	1.648	0.616	0.648	0.071
Lebanon	Jazzine, Pont Al Khalass	0.664	6	3.9	1.654	0.563	0.654	0.132
Lebanon	Mtal al Azraq	0.649	24	5.5	1.649	0.646	0.649	0.009
Lebanon	Wataweet cave	0.662	15	5.5	1.661	0.584	0.622	0.044
Lebanon	Amchite cave / Saleh cave	0.663	11	5.0	1.662	0.584	0.622	0.016
Lebanon	Antelias, Kanaan cave	0.648	9	4.3	1.644	0.584	0.625	0.096
Jordan	Kufranja, Iraq Al Wahaj cave	0.676	12	5.2	1.674	0.590	0.626	0.024
Jordan	Iraq al Amir	0.682	27	7.0	1.682	0.592	0.629	0.027
Jordan	Wadi Dana, cave	0.742	12	6.3	1.742	0.599	0.631	-0.024
Egypt	Kahira, botanical garden	0.626	6	3.8	1.623	0.604	0.634	0.072
Egypt	Asuan, gardens	0.657	22	5.9	1.656	0.604	0.636	0.070
Egypt	El Aquaba	0.648	17	5.2	1.648	0.607	0.637	-0.021
Egypt	Dachla El Qasr	0.571	45	5.5	1.571	0.610	0.641	0.071
Egypt	Mut, Dachla	0.597	10	4.0	1.594	0.607	0.640	0.018
Egypt	Kharga	0.606	17	4.7	1.607	0.606	0.640	-0.081
Oman	Ain Tabruq	0.697	7	5.5	1.696	0.603	0.638	0.027
Oman	Taiq cave	0.739	9	6.2	1.735	0.600	0.637	0.083
Oman	Al Nakhar	0.623	6	3.9	1.637	0.595	0.636	0.081
Unknown	Prague ZOO	0.646	22	4.6	1.644	0.552	0.644	0.140

3.2 Population structure

3.2.1 Individual-based method

Bayesian clustering of the dataset counting 553 genotypes (i.e. dataset excluding Prague ZOO) discloses a distant position of East African individuals and further substructure of the northern part of the species range (see Fig. 2). A pronounced separation of Cypriot population can be seen already from K=2. This K is suggested by Evanno method as the most appropriate (see Fig. 3) (Evanno et al. 2005). For K=3 animals from southern Jordan, Sinai and southern Arabian peninsula cluster together with Iranian and East African ones. From K=4 the latter mentioned group partitions into two, where the representatives from Egypt (except for Sinai) and Sudan form a new subgroup. However, some of the bats from Nile basin seem genetically closer to Levantine assembly, whilst isolated demes from Saharan oases show sole status. The populations from Levant (Syria, Lebanon, Israel, north Jordan) and Turkey create relatively homogeneous group. When dividing into 5 clusters, we receive a result most supported by the estimate of the posterior probability (Fig. 4). From this K on, east African individuals gather together separately from all the others. The above described pattern stays almost identical for K=6 to K=10, only the populations from eastern Oman with those from Iran gradually indicate separation. The estimation of clusteredness (the condition of being clustered) of all individuals is very high for all K signifying high accuracy of the used method (Fig. 5).

The process described above is illustrated by a diagram in Fig. 6. In the diagram, each horizontal line represents a single individual and particular clusters are distinguished by colours. The probability that an individual belongs to the respective cluster is expressed by means of the proportionality of colour sections in each line. For better comprehension of the results from the phylogeographical point of view, genetic structure of every population, based on sum of posterior probabilities of each individual, is set into a map for K=2, 3, 4 and 5 in Fig. 7. The populations are illustrated as pie charts where each sector of a pie represents the probability of belonging to a cluster of its colour.

The total dataset including samples from Prague ZOO does not demonstrate a confirmed genetic relation of these animals to any of the other individuals from our sampling sites. The whole Prague population separates with the same probability of belonging to populations from Cyprus and east Africa as to the rest of the specimens for K=2, then it clusters with new subgroup formed of east African, south Arabian along with Iranian, south Jordanian and Sinai populations for K=3. This allocation receives the highest value of delta K whereas K=6 described below is best supported by posterior probability. The latter mentioned group splits for K=4, so that samples from Egypt separate and new cluster is formed in the same way as in the previous dataset. The grouping for K=5 is almost identical with the one for K=4, except for all animals from the ZOO, which create a new subgroup, and four African individuals which differentiate from the others from their countries of origin and appear more related to the new assembly. For K=6 east African apart from the only Ugandan fruit bat separate completely from other natural populations as well as from Prague collection which segregates as a homogeneous subgroup. The only representative from Uganda shows the same probability of belonging to each of these new clusters and remains unclearly classifiable for every higher K. Graphical displays for visualisation of the results may be found in part II of the Appendix. For K=7 to K=10 the distribution of clusters repeats the results from the former dataset for K=6 to K=9 respectively, therefore the corresponding graphics are not included.

Fig. 3 Mean ΔK versus K

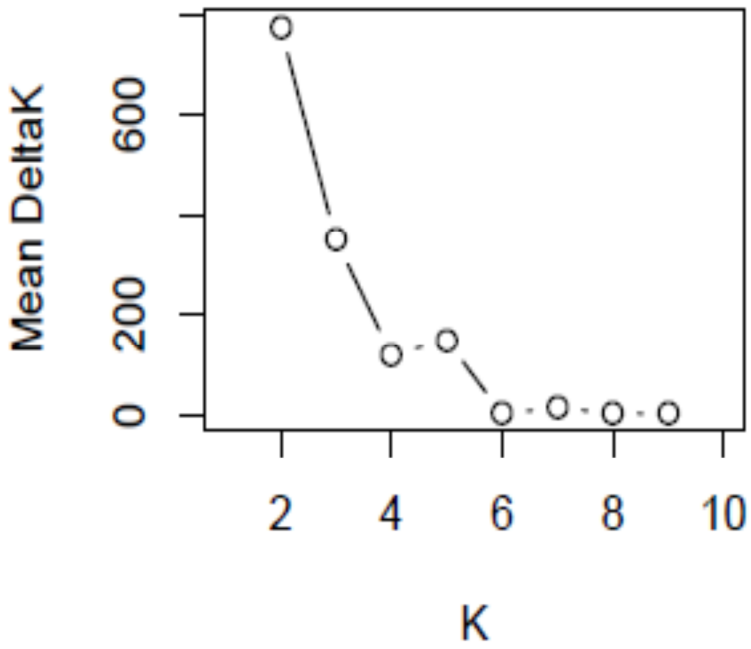


Fig. 4 Estimates of the posterior probabilities for every K

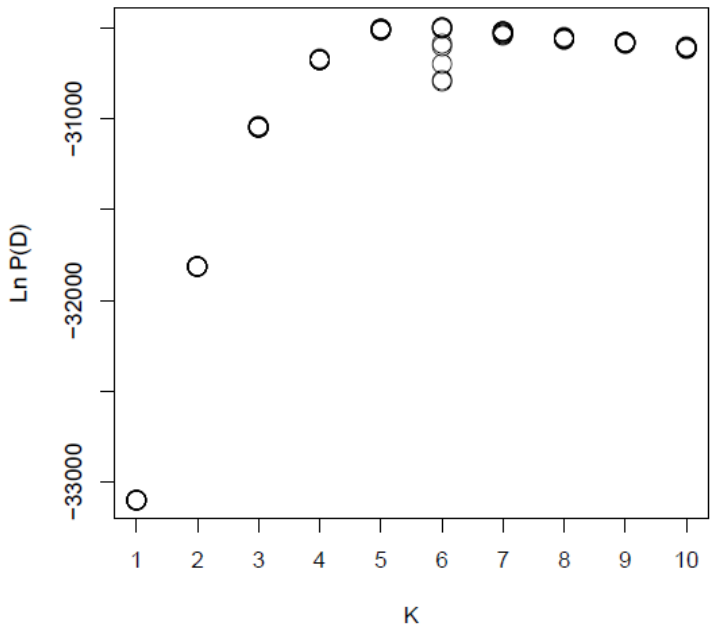


Fig. 5 Clusteredness for every K

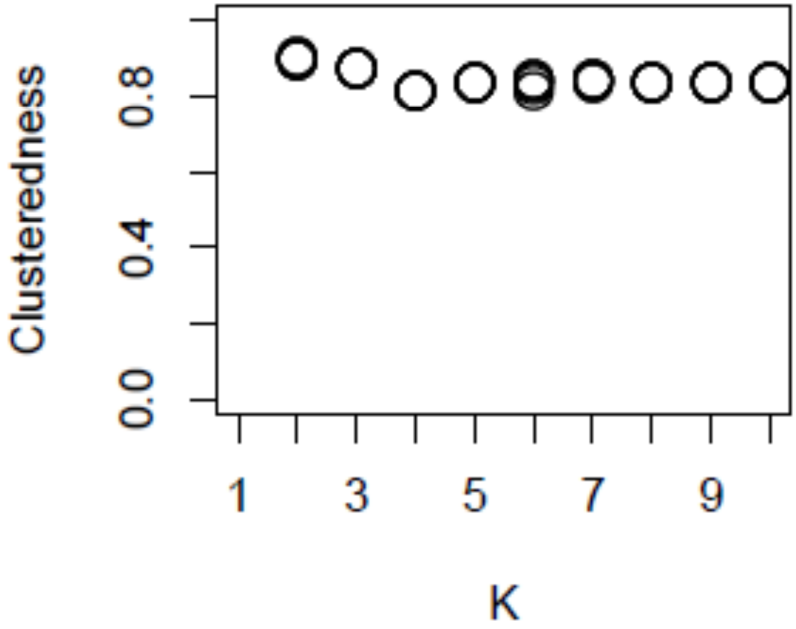


Fig. 6 Graphical display of results from Bayesian clustering

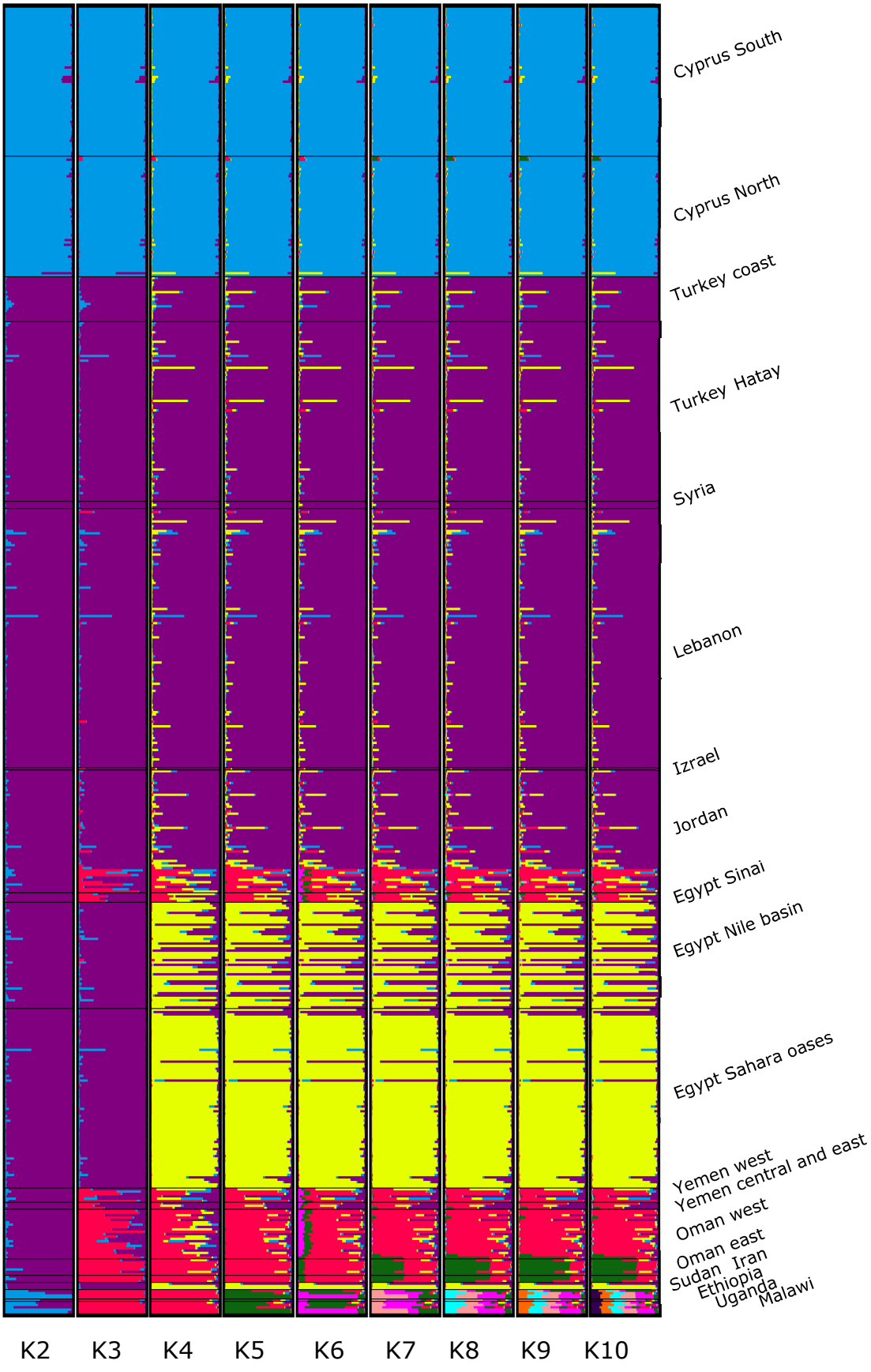
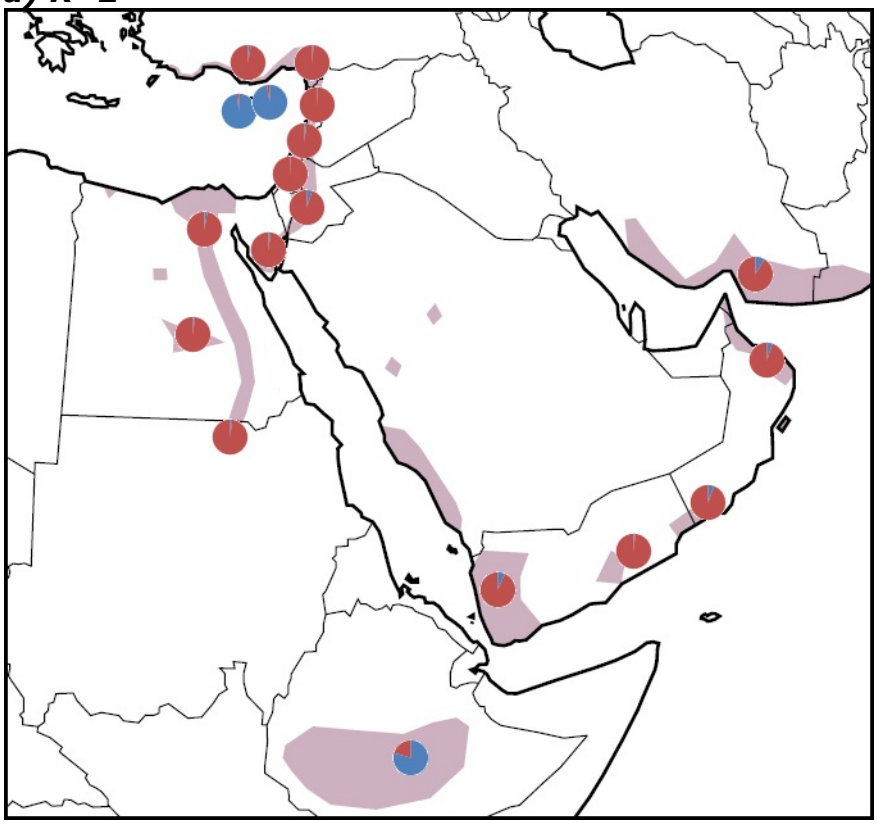
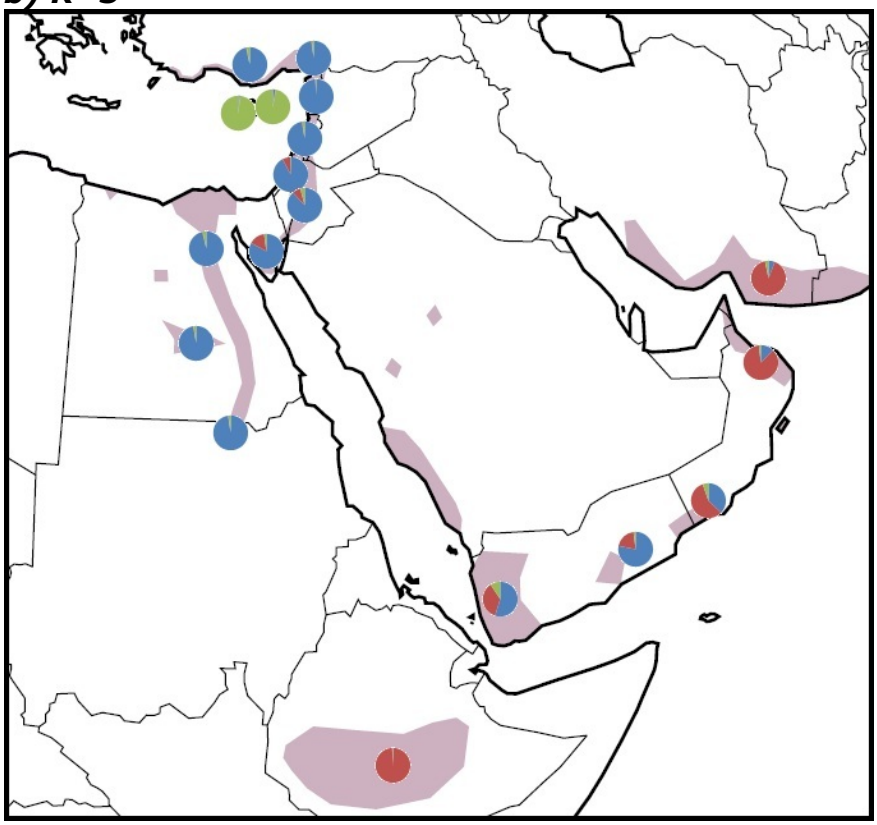


Fig. 7 Maps illustrating the probability of populations belonging to given clusters for a) K=2, b) K=3, c) K=4 and d) K=5

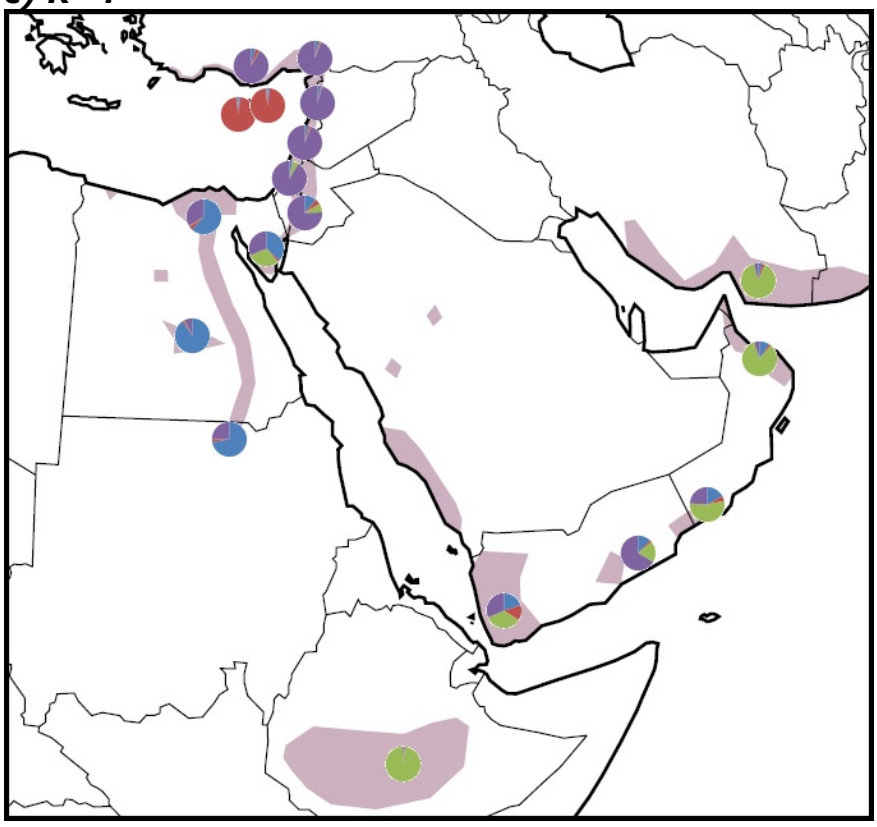
a) K=2



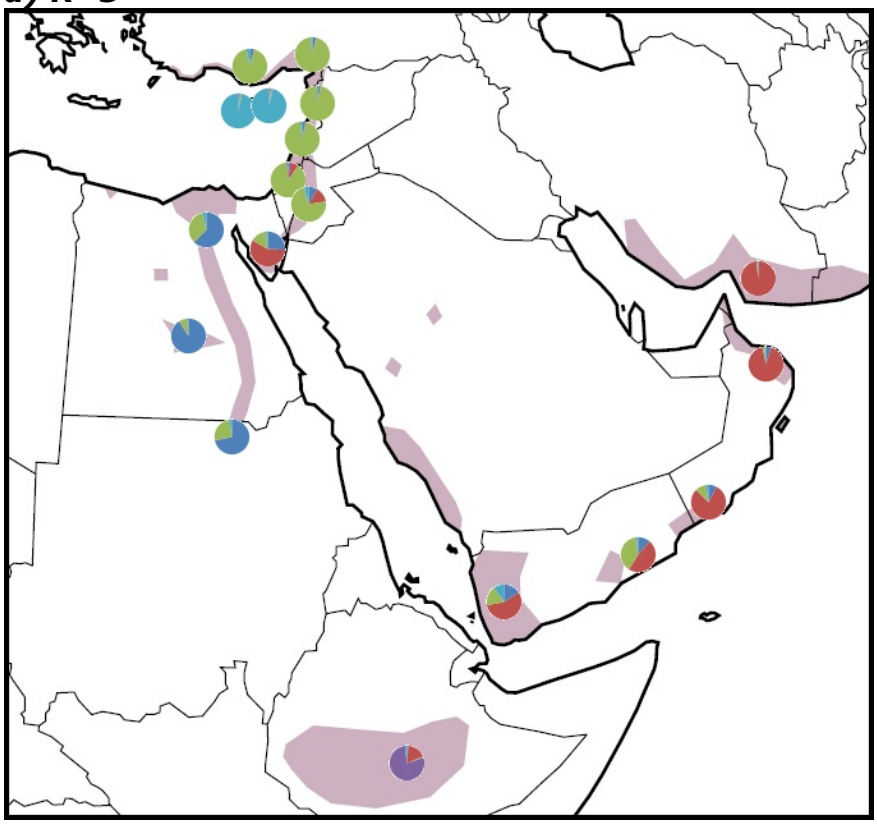
b) K=3



c) K=4



d) K=5

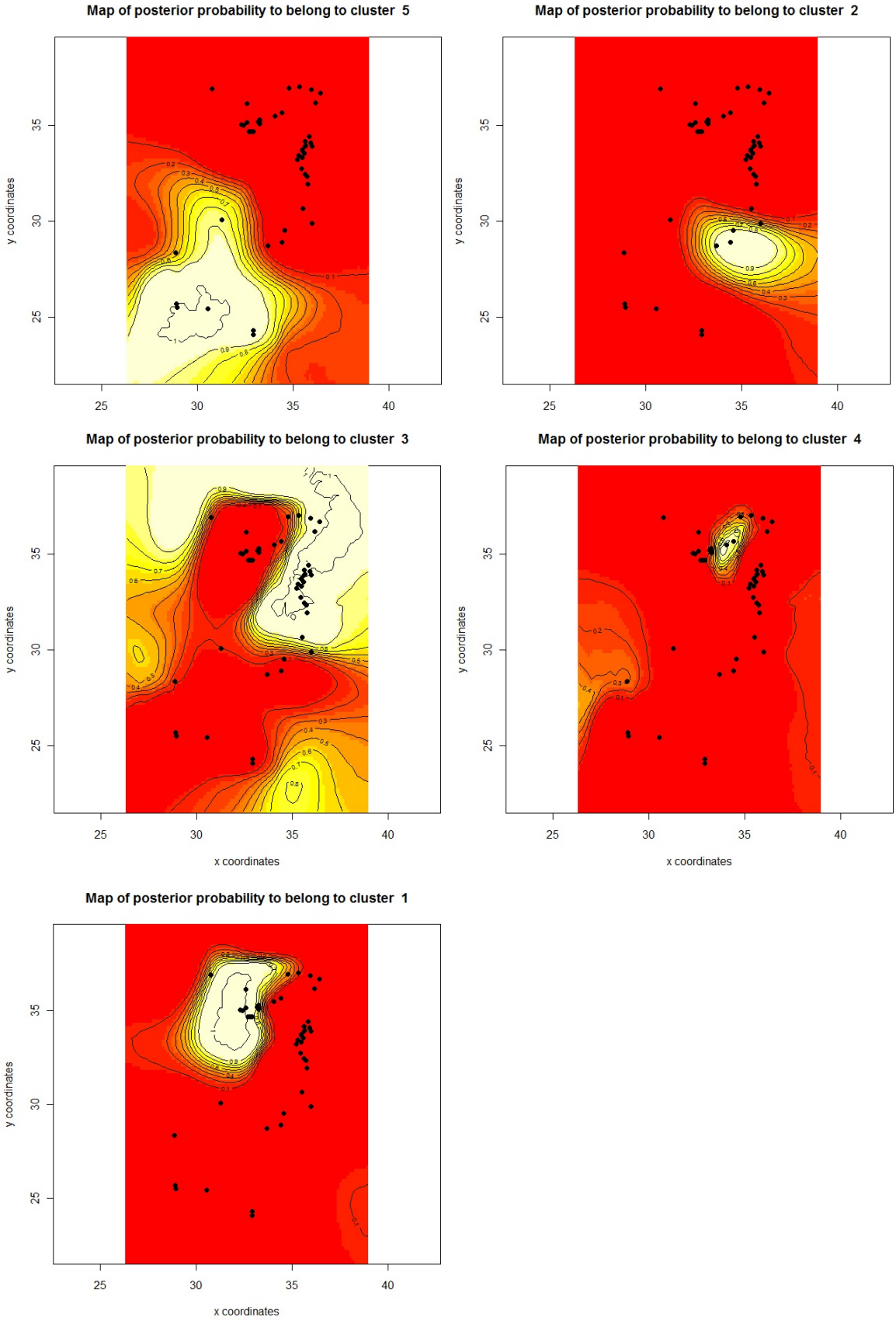


3.2.2 Landscape genetics

When geographical information is involved, we can study our data with landscape genetics approach. Without requiring identification of discrete populations in advance, this method analyses spatial genetic data and allows us to learn how genetic variation is arranged by geographical and environmental features.

Results from the program GeneLand reveal similar grouping within the species as does Structure regarding the northern part of its range. Both programs distinguish spatial domains embracing populations from Nile basin and Sahara oases in Egypt (Fig. 8, cluster 5) Sinai and south Jordan (cluster 2) and Levant, including north Jordan, Israel, Lebanon, Syria and southeast Turkey (Hatay province) (cluster 3). Little difference can be seen regarding Mediterranean Turkey coast and Cyprus. The Turkish southernmost colony, which is also the closest to Cyprus, is coupled with west Cypriot animals (cluster 1) by GeneLand and the colonies from the easternmost part of Cyprus – the Karpas peninsula - are separated (cluster 4).

Fig. 8 Maps of posterior probabilities of population memberships from GeneLand



3.2.3 Isolation by distance

There is a pronounced linkage between geographic and genetic distance among population pairs implied from our data. Plotted results of the examined relationship report direct correlation (Fig. 9, Fig. 10) and statistical significance is assessed by Mantel test (for genetic distance: Z=23581104.9530, r=0.2968, p < 0.001; for Rousset's distance measure: Z = 26230911.2280, r = 0.2821, p < 0.001) (Manly 1994). The latter stated results show that a pattern of isolation by distance (Wright 1943) is present and hence suggests the studied habitat is larger than the average dispersal distance of an individual. Most of the pairwise F_{ST} values range from 0.05 to 0.25 which is deemed to indicate moderate genetic differentiation. Values of pairwise Φ_{PT} , an analogue of F_{ST}, along with probability values based on permutation test are in part IV of the Appendix.

Fig. 9 Genetic distance (F_{ST}) versus geographic distance (m)

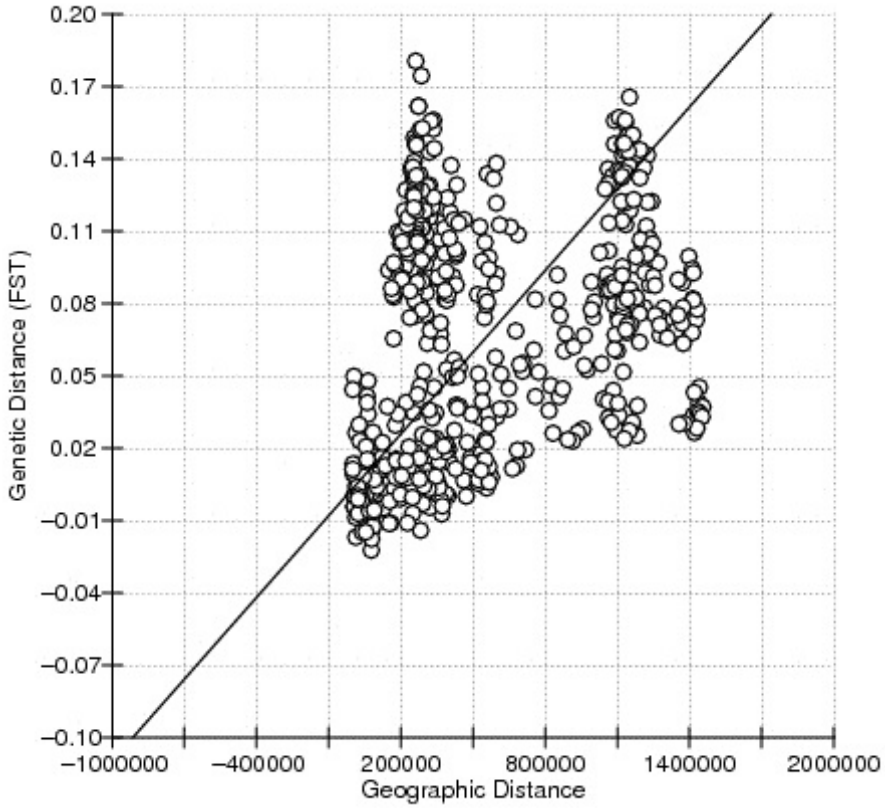
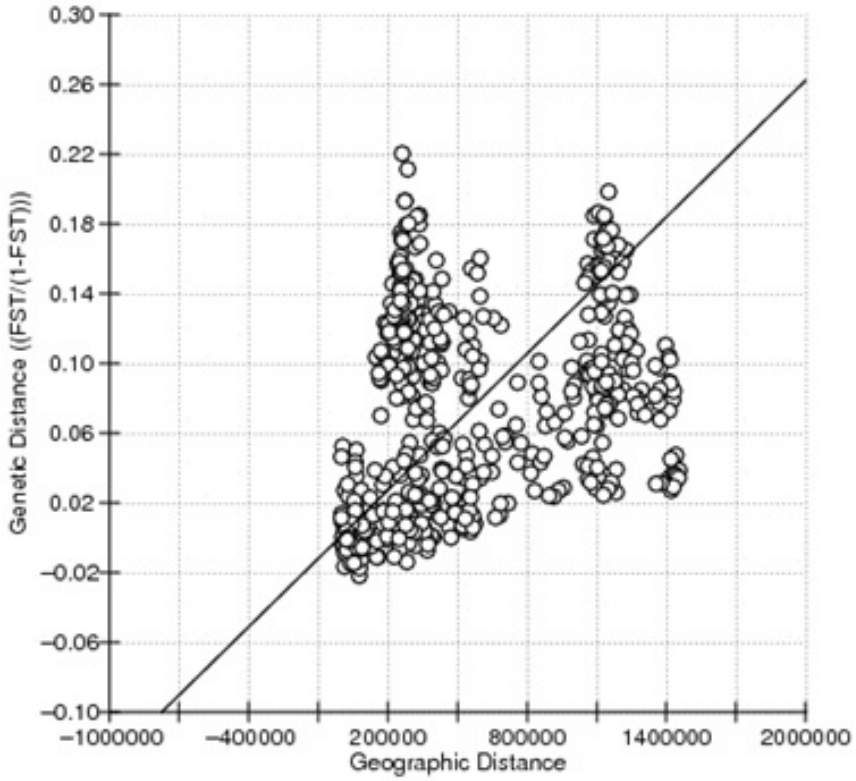


Fig. 10 Rousset's distance measure ($F_{ST}/(1-F_{ST})$) versus geographic distance (m)



4 Discussion

4.1 Taxonomy versus genetics

The most commonly used taxonomy of the genus *Rousettus* happens to be the one of Bergmans (Bergmans 1994). The genus embraces several species involving *Rousettus aegyptiacus* and that is further subdivided into four subspecies: *R. a. aegyptiacus* (east Mediterranean region), *R. a. arabicus* (southeast Arabian peninsula, Iran, Ethiopia), *R. a. leachii* (east Africa) and *R. a. unicolor* (west and central Africa). All subspecies are believed to inhabit ranges which do not overlap (Bergmans 1994). In accordance with Juste et al, other two subspecies which live on two islands in the Gulf of Guinea vary from *R. a unicolor* – the closest mainland subspecies (Juste et al. 1996).

Consistent with our data resulting both from Bayesian clustering and landscape genetics, animals from the Mediterranean region (*R. a. aegyptiacus*) and those classified as *R. a. arabicus* do not differ in the way previously presumed. The uncovered subgrouping, *inter alia,* suggests that Egyptian fruit bats from Yemen, Oman and Iran are closely related to Sinai ones. Although studies based on mitochondrial DNA (Benda et al. 2007; Benda et al. 2012 in prep.; Dundarova 2011) do not prove very marked genetic heterogeneity among the whole area we have studied, skull dimension comparisons and overall morphology are consistent with our outcome (Benda et al. 2008).

The separate position of Cypriot populations recalls the situation in the Gulf of Guinea or in the Indian ocean eastwards from Africa. The insular representatives of the genus *Rousettus* are denominated as subspecies of the species *Rousettus aegyptiacus* in the Atlantic (*R. a. tomensis* and *R. a. princeps*) but as discrete species on Comoro islands and Madagascar (*R. obliviosus* and *R. madagascariensis* respectively) in the related studies (Juste et al. 1996; Goodman et al. 2010). However, values of genetic distances of mitochondrial genes among Mediterranean populations (Benda et al. 2007; Dudarova 2011) are much lower (0.1–0.4 per cent) compared to island versus mainland Rousette populations in the Indian ocean (8.5–13.2 per cent; Goodman et al. 2010). Moreover, the sister species

to *R. madagascariensis* and *R. obliviosus* clade has neither been well determined nor has *R. aegyptiacus* been defined as the most likely. Unfortunately, comparable haplotype diversities for the two subspecies mentioned above (*R*. *a*. *tomensis* and *R*. *a*. *princeps*) are not available but genetic differentiation based on allozyme electrophoresis manifested the mainland and both island populations to be clearly detached (Juste et al. 1996). Consequently, tendency for island speciation among fruit bats is apparent and satisfactorily supported by the fact that 40 per cent of the species of the *Rousettus* genus are island endemics.

Recent reputable studies have already proved the uniformity of morphological measurements shared by animals from Turkish and Levantine populations (Karatas et al. 2003; Benda et al. 2008). Our results fully sustain the evidence genetically.

In any case, the genetics does not encourage previous taxonomy in grouping all Mediterranean individuals into one cluster. Moreover, it proves the two original subspecies thought to be separated geographically probably deploy a different spatial distribution and that the Egyptian fruit bats in their northernmost part of the range show more complex population pattern. The insignificant depth of the divergence between clusters resulting from mtDNA may be explained by recent expansion of the species as also supported empirically in the respective study (Dundarova 2011).

4.2 Potential geographical barriers to gene flow

The importance of geographical barriers as potential restrictions for gene flow is obvious as these represent diverse difficulties for animals to go across. In our findings, we can see three main locations where two different clusters split, and therefore it appears to be relevant to study the respective landscape to find out more about the environment in such areas. Despite the fact that the population substructure inferred by using two independent methods revealed very similar results, each corresponding partition is discussed individually to stress the limitations to gene flow in both possible perspectives.

4.2.1 Population assembly by means of Bayesian probability

The first and most implying separation lies between the south Turkish coast and Cyprus. Geographical barrier is created by the Mediterranean sea here and the closest distance for fruit bats to fly over is around 70 km. Goodman et al. (2010) have indicated that water expanses of 13 km is being easily traversed by *Rousettus madagascariensis* whilst there is evidenced gene flow from mainland of Madagascar to neighbouring islands. Furthermore, a considerable migration of *Rousettus obliviosus* among Comoro islands has been proven by genetic structure inferred from survey of mitochondrial and nuclear markers. These islands are divided by a water barrier of up to 80 km (Goodman et al. 2010). On the other hand, based on allozymes, Juste et al. (1996) proposed *R. a. tomensis* and *R*. *a*. *princeps* to become denoted as isolated subspecies rather than members of *R. a. unicolor*. The remarked subspecies live on two islands in the Gulf of Guinea (Sao Tome and Principe), which have only 146 km of distance in between and from each island to the mainland 280 and 220 km respectively (Juste et al. 1996). Hence, the reason for limited gene flow between coastal and insular populations of *Rousettus aegyptiacus* in the Mediterranean seems not to be determined only geographically.

The second border of two putative clusters has formed somewhere in between of our two Jordan populations. The situation is much more complicated in this area because the distinguished colonies are situated 144 km from each other and there are more colonies in their connecting corridor we do not have samples from. Despite the stated lack of clarity, when looking at the species range of other bats inhabiting Jordan (Benda et al. 2010), we may derive that the Dead sea is the most marked unevenness, and thus possibly play the role of a geographical barrier. As graphically depicted in the last cited study, many bat species either live southwards from the Dead sea and are not to be found northwards or vice versa.

The third boundary occurs approximately on the margin of Sinai peninsula and disconnects Nile populations from the Sinai/south Jordan ones. The border of the latter subgroups could be further extended alongside the south coast of Arabian peninsula when populations from Yemen are taken

into account. From Sinai to the west, there is obviously the main obstacle created by the Red sea, whereas to the north, the main reason for fruit bats not to migrate could be given by the presence of relatively broad expanse of mountains offering severe living conditions. Alike the other Middle Eastern fruit bats, the populations from Sinai have probably been established quite recently after the settlements of people who started to cultivate these arid zones (Benda et al. 2008). Thus, the question here should be where they did come from rather than where their limits for expanding further are. The clear genetic and morphologic relationship with animals from southeast of Arabian peninsula evokes the idea of their origin in these areas. However, vast land which spans between the regarded populations bears no evidence of fruit bat colonies.

Various explanations could be applied to resolve the oddity. Either the absence of fruit bats on the north coast of the Red sea is an observation artefact and it is just a matter of time to discover these concealed colonies or the bats traversing such an extreme distance form only temporary colonies on their way. As the gap stretching between the two closest recorded roosts makes approximately 600 km, we may assume the fruit bats have capacity to overcome even that long flight with some frequency. This slightly unexpected relatedness of populations that are so geographically distant emphasises the meaningfulness of molecular analyses exposing facts hardly traceable by other methods.

Gradual dissimilarity may be also seen between the populations from Nile basin in Egypt and Sudan and the colonies residing in the western oases. From the geographic point of view the local barrier for gene flow is created by desert, an environment unlikely to sustain major migration. Additionally, all colonies residing in the Nile basin even as far as the one from Sudan revealed relation to the Levantine populations, and hence the data support occurrence of recent or sub-recent gene flow along the stream and further along the coastline.

4.2.2 Subdivision via landscape genetics

There are only two minor discrepancies between the two methods used. Thus, except the below specified findings, subpopulations revealed by

GeneLand are congruent with the previous technique and delineated in paragraph 4.2.1.

In spite of the abundance of *Rousettus aegyptiacus* populations inhabiting Cyprus, their distribution is surprisingly incoherent (Benda et al. 2007). Relatively noticeable separation of Karpas peninsula populations is present and the results obtained from GeneLand program encourage an idea that this part of the range is affected by processes acting in small populations.

On the other hand, fruit bats from the closest location on the mainland Turkey seem to be more associated with south Cypriot cluster rather than with other Turkish colonies. This southernmost Turkish colony is situated on a foreland and the reason for it being more related to southern Cyprus might be assessed when our results from microsatellite genotypes are compared with outcomes based on mitochondrial sequence data. On the maps resulting from mtDNA sequences animals from Cyprus belong to the same cluster as all colonies from the Turkish coast but Levantine ones (Dundarova unpubl. data). According to the difference in mutation rate between the two molecular tools, this contrasting pattern may point to the direction of genetic changes in time.

4.3 Colonization and limits to dispersal

The current distribution of *Rousettus aegyptiacus* and particularly the fact it inhabits seasonally cold and relatively dry zones directs us to a possible connection between the species dispersion and the beginning of cultivation of domestic plants in the Mediterranean areas which correlates with a recent population growth of the species (Benda 2008; Dundarova 2011). In 1976, Galil already suggested that the invasion of fruit bats into the region was a consequence of cultivation of *Ficus sycomorus* by men (Galil et al. 1976, as cited in Korine et al. 1999). In addition, the genus *Ficus* composes a major proportion of the diet in Israel (Korine et al. 1999). On the contrary, in Turkey the bats feed mainly and year-round on *Melia azadirachta* (Persian lilac) and on Cyprus *Ceratonia siliqua* (carob) seems to be the most frequent food (Benda 2007). In any case, plantations, fields or gardens represent a great target for foraging fruit bats despite the displeasure of indigenous farmers. In view of the reported diet of local populations, their

feeding resources are well supported by the agricultural crops (Albayrak et al. 2008). Still, while feeding on both wild and commercial plants, only ripe fruit is being selected and so the impact on the productiveness of many crops should be minimal as most fruits are harvested before the mature stage for transportation (Hadjisterkotis 2006).

As flying mammals, the ability to disperse may seem to be considerable for bats, however, due to the unstable climatic conditions, there are only limited suitable roosts of fairly constant temperature and humidity like caves, rock crevices and ruins. Additionally, it has been proved for many other bat species on the example of the narrow Strait of Gibraltar that the flight capacity does not necessarily have to correlate with the fact bats actually do or do not fly over certain barriers or distances (Garcia-Mudarra et al. 2009). Since no population structure of another *Rousettus* from India and south China has been proven by molecular analysis, not only cave dwelling but also the climate must play an important role in the inclination to dispersion.

Colonies of *Rousettus leschenaulti* have been examined by means of both nuclear and mitochondrial markers in southeast and east Asia with the most distant locations divided by approximately 1500 km. Despite the enormous span of the study region, genetic homogeneity was detected indicating high level of gene flow among all colonies of the species (Chen et al. 2010). The authors highlighted the consequences of behavioural variations when comparing well-structured populations of tree roosting species with the cave roosting *R. leschenaulti* in the area, whereas we may see their findings from a different point of view. Among species of the same genus, fruit bats inhabiting tropical areas (*R. leschenaulti*) tend to show high degree of vagility, while those populating regions with moderate climate (*R. aegyptiacus*) do not always behave in this way. In the light of the observations mentioned above, the fact our resultant genetic clusters are either covering a small well-defined geographical domains or expansive territory may imply the population structure of Egyptian fruit bat is also influenced by philopatry or diverse, hardly measurable, social behaviour in the respective areas.

Regarding the population size and local abundances, the biggest threat for the species are acts of humans who often spray, shoot, fumigate or blockade caves in order to eliminate fruit bats considered as agricultural pests. Similar effects are connected with urbanization leading to destruction of old houses, which may accommodate bat colonies (Alabaster 2012; Albayrak et al. 2008; Amr et al. 2006). Many species are actually hunted for food or sport and sold as a delicacy, which has caused their rapid decline in several countries (Fujita and Tuttle 1991). Hence ironically, people in the Mediterranean, who probably enabled fruit bats to populate their current range, indeed represent an enormous threat of eradication to them.

4.4 The origin of fruit bats from Prague ZOO

According to Bayesian clustering we may assume the animals from Prague ZOO are not related to any of the natural populations sampled in our study as they separate from all other subgroups from K=6 on. Although the group stays close to part of Malawian and part of Ethiopian animals for K=5, we would need to analyse more samples from both of the regions to support this questionable parting of east African rousettes. Especially when other specimens from these countries cluster with the south Arabian/Sinai populations for the same K. Considering their isolation in the ZOO, the genetic distance might develop due to founder effect and small population size causing high level of mating between related individuals. However, our results do not support the idea, as the inbreeding coefficient value is not that high (0.14). Moreover, the calculations of H_E and H_O do not differ substantially from corresponding values counted for natural populations. Therefore, we believe that these animals come from areas we have not sampled and potentially different *Rousettus* species has largely contributed to the population.

5 Conclusions

Microsatellite loci were examined in our research to find out whether fruit bats from the northernmost part of their distribution range, represented by the single species *Rousettus aegyptiacus*, exhibit genetic pattern associated with geographical location. The previous studies focused on genetic diversity of the Egyptian fruit bat in this specific area have provided ambiguous results. They either denied any population substructure in the region and challenged the current taxonomic division into two subspecies or supported the presence of discrete clusters yet without profound divergences between them. Until now, no other molecular marker has been used, except for mitochondrial DNA sequences. Although once denied as a result of former analyses, the presence of evident population structure of *R. aegyptiacus* in the Middle East seems to be proven now.

The reasons for constrained gene flow, and thus creation of separate clusters, include difficulties in crossing certain obstructions in the landscape, in particular wide expanses of sea, deserts or high mountains. Apart from geographical barriers, other limiting factor for dispersion might be the lack of suitable roosting sites in the area. Given the capability to fly for long distances, also site fidelity of the animals and their feeding and roosting ecology ought to be considered. Potential philopatry could be implied from the significant results of isolation by distance model.

In contrast to the existing taxonomic subdivision of the Egyptian fruit bat in the respective region, the presented results show a more differentiated composition of closely related populations. The current subspecies *R. a. aegyptiacus* encompasses all Mediterranean populations, whereas *R. a. arabicus* should inhabit distant territories in the southeast of Arabian peninsula, Ethiopia and the south of Iran and Pakistan. Our data hereby question the current view by revealing outcomes of genetic analyses that presume (i) relatedness of animals from Sinai and south Jordan with southeast Arabian colonies, (ii) profound separation of Cypriot populations, (iii) sole status of fruit bats from oases in Egypt, and (iv) gradual disjunction of east Oman together with Iran populations from their neighbouring colonies. Some uncovered clusters are well supported by other

relevant studies. For example, the isolation of insular populations occurs quite frequently among fruit bat species and it is only the level to which the animals from islands are genetically distant from the mainland populations that varies. Likewise, the relationship between Sinai and southeast Arabian fruit bats has been observed when morphological traits were compared. Finally, although separated by shallow divergences, similar solitary subgroups were discovered when data from mtDNA sequences were diagnosed by landscape genetics approach.

A possibility of causal connection between the beginning of planting agricultural crops by mankind and the invasion of fruit bats to the Mediterranean basin has been hypothesized. However, the correlation of population expansion of *R*. *aegyptiacus* with the humans' initiation of cultivation, no matter how apparent it could seem, is hard to prove unequivocally. On the other hand, it is clear that humans have largely contributed to the decline of the species by inconsiderate acts in order to provide protection of their yield.

A collection of 22 Egyptian fruit bats from Prague ZOO was included in additional analyses in order to infer their origin by comparison with individuals from natural environment. None of sampled colonies indicated to be genetically close to these specimens and we suppose three possible reasons for the separation of the Prague bats exist: (i) we do not have samples from the area of their origin, (ii) they were strongly influenced by bottleneck demography or (iii) they are crossbred with another species.

As a final point, our records embody the beginning of a complex research of the subject, which seems to be interesting, as the widely accepted taxonomy of the examined species is contradicting our findings. Following analyses may concentrate on a more detailed study of the relationship between northern populations of *R*. *aegyptiacus* and east African subspecies. In addition, by including larger number of samples from Jordan, more accurate determination of the suture zone in that area would be elucidated. Finally, the apparent gradual separation of Cypriot populations, which could lead to island speciation, attracts our attention and this location should be investigated more thoroughly.

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